Assessing *Nezara viridula* (Hemiptera: Pentatomidae) Feeding Damage in Macadamia Nuts by Using a Biological Stain

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ABSTRACT Damage caused by southern green stink bug, *Nezara viridula* (L.), to macadamia nuts, *Macadamia integrifolia* Maiden & Betche, is normally determined after nuts are harvested and processed, which may be many months after damage occurred in the field. We developed a method using ruthenium red dye to stain stink bug feeding probes and indirectly assess feeding activity in macadamia nuts. By using the staining method, feeding probes were easily detected on the husk, shell, and kernel. Husk probing was highly correlated (0.80–0.90) with feeding and damage to the kernel. Failure rate to detect kernel damage from stained husk probes was generally <6%. The staining method was equally effective for immature and mature nuts; therefore, *N. viridula* feeding activity can be monitored throughout the season to evaluate pest management tactics and forecast outbreak populations.

KEY WORDS southern green stink bug, monitoring, salivary enzyme, dye, IPM

Macadamia nut, *Macadamia integrifolia* Maiden & Betche (Proteaceae), is the largest orchard crop grown in Hawaii with 18,000 acres in production and a total farm value of $30–40 million (HASS 2005). *Nezara viridula* (L.) is one of the main insect pests of macadamia nuts. Typically, *N. viridula* causes industry-wide damage of <2% annually (HASS 1990–2000). This level of damage is considered to be economically tolerable by the industry and normally insecticides have not been applied for *N. viridula* control. In 2002–2003, crop losses because of *N. viridula* increased to 3.5% for unknown reasons (HASS 2004). In some orchards, *N. viridula* damage levels exceeded 50% (M.G.W. and P.A.F., unpublished data). Damage was high again in 2004–2005 as 3.7% of the delivered crop was rejected due to *N. viridula* damage (HASS 2005). Actual losses because of *N. viridula* may be considerably higher because feeding also causes premature drop, resulting in immaturity, and the introduction of pathogens causing moldy nuts, both of which result in rejection at the processor. Several growers with large acreage have begun applying insecticides to control *N. viridula* when large numbers of the stink bugs are observed on broadleaf weeds in the orchard. No other species of stink bug or other Hemiptera attack macadamia nut in Hawaii (Jones 2002).

Damage occurs to the nut when *N. viridula* places its stylet-like mouthparts on the nut husk and secretes saliva containing a suite of digestive enzymes that softens the husk, allowing it to insert its mouthparts through the husk and shell to the kernel. *N. viridula* then digests an area of the kernel (Mitchell et al. 1965), leaving a discolored pit on the kernel surface (Fig. 1). *N. viridula* will feed on any age of nut, although laboratory studies suggest *N. viridula* prefers mature nuts to immature nuts and nuts with green rather than brown husks (Shearer and Jones 1996). In addition to damaging kernels, *N. viridula* feeding has been shown to cause premature drop (Jones and Caprio 1994), resulting in immature nuts being harvested from the ground (Bittenbender and Hirae 1987). Also, molds and fungi may enter the nut and infect the kernel via the *N. viridula* puncture wound (Jones and Caprio 1990).

Macadamia trees have indeterminate flowering, and the time from anthesis to nut drop is ≈30 wk (Nagao and Hirae 1992). The main period of harvest is August through January (multiple harvests), but stink bug feeding may occur throughout the year on either developing nuts or mature nuts. Macadamia is not a preferred food plant for *N. viridula* (Shearer and Jones 1996). *N. viridula* feeds on leguminous and cruciferous weeds in macadamia orchards and moves on to macadamia nuts in the tree and on the ground when weeds begin senescing (Jones et al. 2001) or are controlled by mowing or herbicide use (Jones 2002). Previous studies suggested that when *N. viridula* feeding damage occurs on the ground, it occurs mainly in the first week after the nut falls (Jones and Caprio 1994). Feeding damage also occurs in the tree canopy throughout nut development, suggesting that it is important to manage *N. viridula* within macadamia tree canopies and on the ground.

To effectively implement integrated management of *N. viridula* in macadamia orchards, it is important to

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Fig. 1. Typical damage to macadamia kernel caused by *N. viridula* feeding.

Fig. 2. Stained probe to husk exterior of a maturity class 4 nut (original magnification, 6×).

monitor the stink bug numbers and to predict when and whether remedial measures need to be taken. A major drawback in *N. viridula* management is the lack of an effective monitoring technique (Jones 2002, Leskey and Hogmire 2005), an issue we address with this study. Macadamia nut growers in Hawaii have not attempted to monitor stink bug in the past.

Campbell and Shea (1990) demonstrated that ruthenium red dye stains the puncture wounds of the western conifer seed bug, *Leptoglossus occidentalis* Heidemann (Hemiptera: Coreidae), by reacting with salivary enzymes secreted during feeding on cones. The kernel of the macadamia nut is enclosed in a hard shell and husk and cannot be assessed for damage caused by *N. viridula* without extraction from the shell and drying. Developing a method to evaluate levels of macadamia kernel damage without shelling and drying the nut would be of great practical value to rapidly assess feeding activity. We adapted the ruthenium red dye staining technique developed for *L. occidentalis* to mark the salivary enzymes used by *N. viridula* during feeding on macadamia nuts. Studies were conducted to determine the effectiveness of the dye in staining stink bug probes, to test the potential for predicting kernel damage from probing activity to the husk and shell and to evaluate feeding patterns in immature and mature nuts throughout the season.

Materials and Methods

Macadamia nuts were collected from orchards in three distinct growing areas of the Big Island of Hawaii, Kohala (average rainfall 93.6 cm/yr), Kau (average rainfall 60.96 cm/yr), and Hilo (average rainfall 330.2 cm/yr) from April to December 2004. In each orchard block, samples were taken by walking along a transect and randomly collecting 10 immature or mature green nuts from 10 to 30 trees along the transect (n = 100–300 nuts). Nut maturity class was determined using a system devised and applied by MacFarms of Hawaii. Class 1 nuts were collected from six blocks (n = 100 per block), and averaged 16.5 mm in diameter at 8 wk from anthesis (full flowering). Class 1 nuts have a very soft shell, the kernel is immature, and the husk is adhering to the shell. Class 2 nuts were collected from five blocks (n = 200 per block), and averaged 18.6 mm in diameter at 12 wk from anthesis. Class 2 nuts still have a soft shell and an immature kernel, but there is separation of the husk from the shell. Class 3 nuts were collected from four blocks (n = 200 per block), and averaged 26.5 mm in diameter, at 18 wk from anthesis. Class 3 nuts had a hardened shell, a mature kernel, and white-to-tan husk interior. Class 4 nuts were collected from 12 blocks (n = 300 per block) and averaged 31.6 mm in diameter at 24 wk from anthesis. Class 4 nuts have fully mature kernels and the interior husks are light brown to chocolate brown. Class 1, 2, and 3 nuts were collected from the tree, and class 4 nuts were collected off the ground. Samples collected in April and May included class 1 nuts, samples collected in June included class 2 nuts, samples collected in July and August included primarily class 3 nuts, and samples collected after August included primarily class 4 nuts. At least one block from each growing region was sampled for each maturity

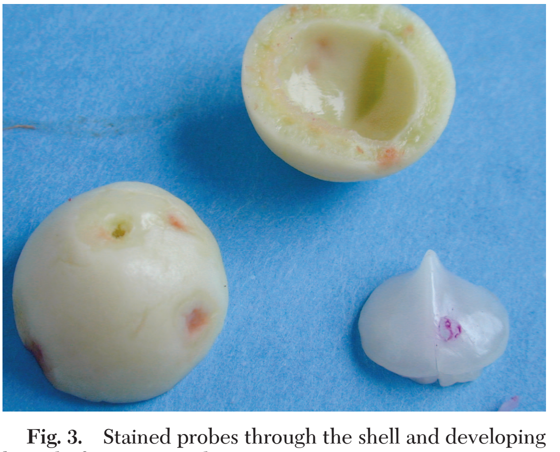

Fig. 3. Stained probes through the shell and developing kernel of a maturity class 1 nut.

Table 1. Average pairwise correlation values between stained *N. viridula* probes at different locations in the macadamia nut and kernel damage

Maturity class	n[a]	No. nuts	Exterior husk × kernel		Interior husk × kernel		Shell × kernel	
			Mean[b]	SEM	Mean	SEM	Mean	SEM
1	6	600	0.84a	0.03	0.86a	0.03	0.89a	0.04
2	5	1,000	0.86a	0.04	0.90a	0.04	0.90a	0.03
3	4	800	0.92a	0.02	0.92a	0.01	0.90a	0.03
4	12	3,600	0.79a	0.02	0.82a	0.01	0.86a	0.02

[a] Number of orchard blocks sampled.
[b] Mean correlation values within columns followed by the same letter are not significantly different ($P > 0.05$) according to a *t*-test.

class. Samples were collected randomly across a patchwork of cultivars, corresponding to a typical grower's sample submitted to the processor for quality control.

To stain feeding probes, freshly harvested nuts were submerged in a 0.05% aqueous solution of ruthenium red in deionized water, shaken for 2 h at room temperature (23 ± 2°C), and rinsed in distilled water for 30 min. Stained probes (≈0.50 mm in diameter) showed up under a stereomicroscope (6× magnification) as circular, magenta pink spots (Fig. 2). Plant tissues contain enzymes to repair abrasions and other wounds, which also bond with the dye, but these stained areas are irregular in shape and larger than *N. viridula* puncture wounds, and they have a deeper red color. In some nuts, the entire exterior husk is roughened and russeted, possibly because of broad mite, *Polyphagotarsonemus latus* (Banks) (Acari: Tarsonemidae) or redbanded thrips, *Selenothrips rubrocinctus* (Hassan) (Thysonaoptera: Thripidae), damage (Jones 2002); the exterior husk of these nuts turns deep red after dyeing, but even on these nuts the magenta probe from *N. viridula* is easily distinguished.

After staining, data were collected on nut size and maturity, and any probe marks on the husk (exterior and interior) and shell (exterior), and kernel damage (evidence of dye or actual pitting), were counted and recorded. The kernels of maturity class 1 and 2 nuts were too immature to oven dry, so damage to the kernel in these nut classes was determined by staining only (Fig. 3). Maturity class 3 and 4 nuts were observed for exterior husk staining, and then husked; data on stained probes from the interior husk and exterior shell were recorded, then nuts were oven-dried at 60°C for 3 d and cracked to assess kernel damage. After drying, stink bug feeding damage to the kernel showed up as darkened craters or pitting on the surface of the kernel.

Data were analyzed as a completely randomized design with orchard blocks serving as replicates. The effect of growing area was not considered because of limited replication within areas. Data on percentage of kernel damage were arcsine transformed and subjected to analysis of variance (ANOVA), and means separations were done using *t*-tests (SAS Institute 2002). Data on stink bug probing to the external husk, internal husk, or shell, and damage to the kernel were subjected to regression and pairwise correlation analysis. Correlation data were normalized using Fisher

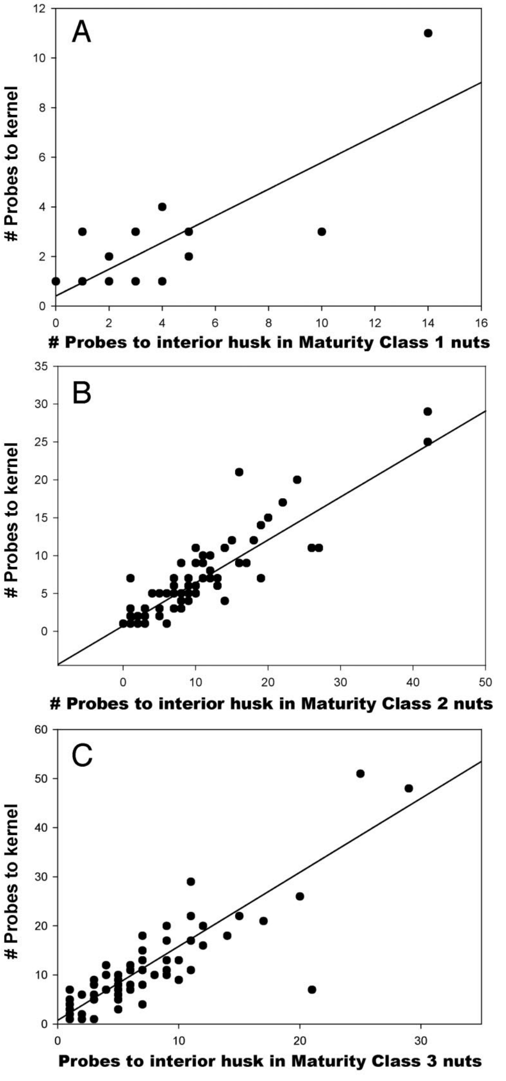

Fig. 4. Regressions of husk probing by kernel pitting in three macadamia nut maturity classes. (A) Interior husk probes by kernel probes in maturity class 1 nuts. Equation for the regression line is $y = 0.41x + 0.54$ ($r^2 = 0.85$, $P = 0.0003$). (B) Interior husk probes by kernel probes in maturity class 2 nuts. Equation for the regression line is $y = 0.50x + 0.63$ ($r^2 = 0.76$, $P = 0.0001$). (C) Interior husk probes by kernel probes in maturity class 3 nuts. Equation for the regression line is $y = 0.54x + 0.64$ ($r^2 = 0.80$, $P = 0.0001$).

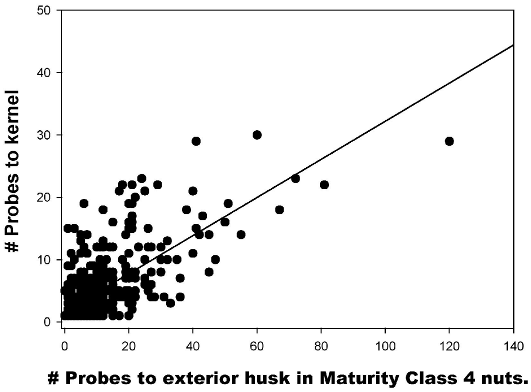

Fig. 5. Exterior husk probes by kernel probes in maturity class 4 nuts. Equation for the regression line is y = 0.72x + 0.57 (r^2 = 0.90, P = 0.0002).

Z_r transformation and then subjected to ANOVA (Steiger 1980, Sheskin 2000) to compare correlation rates among maturity classes; mean separations were done on significant treatments using *t*-tests (SAS Institute 2002). Failure rates for predicting kernel damage from stained probes on the husk and shell were estimated by calculating the percentage of kernels damaged when no external husk probes were detected and when no external and internal husk probes were detected.

Results and Discussion

ANOVA on percentage of kernel damage data were significant for the effect of maturity class (F = 3.19; df = 3, 23; P = 0.043). On average, 13.0, 9.6, 23.8, and 21.9% of class 1, 2, 3, and 4 nuts had probes to the kernel. Damage to class 3 and 4 nuts was significantly greater than damage to class 1 nuts, whereas damage to class 2 nuts was intermediate and not significantly different from class 1, 3, or 4 nuts. The various classes of nuts were collected at different times during the season and therefore differences in damage do not necessarily indicate differences in *N. viridula* feeding preference. Although late season (class 3 and 4) nuts showed numerically higher damage than early (class 1 and 2) season nuts there was no clear trend suggesting damage accumulated on the tree. The appearance of lower damage early in the season compared with later in the season may be due to the tendency of damaged immature nuts to drop prematurely (Jones 2002). Nevertheless, *N. viridula* probes stain clearly even after several months' time (M.G. and P.A.F., unpublished data); therefore, probes detected in mature nuts may have been from stink bug feeding early during the development of the nut.

Correlations were consistently high between kernel damage (pitting) and external husk (r = 0.79–0.92), internal husk (r = 0.86–0.92), and external shell (r = 0.86–0.91) staining for nuts in all maturity classes (Table 1). This indicates that stained probe marks on the husk and shell are good predictors of kernel damage within for all nut maturity classes. ANOVA on correlation data were not significant for the effect of maturity class for the exterior husk by kernel correlations (F = 2.73; df = 3, 23; P = 0.067), the interior husk by kernel correlations (F = 2.33; df = 3, 23; P = 0.10), or the shell by kernel correlations (F = 0.83; df = 3, 23; P = 0.49) (Table 1).

In class 1, 2, and 3 nuts, probes to the exterior often overlapped each other and were not distinguishable as separate probes, but they were inferred by the obvious presence of probes to the interior husk at positions corresponding to suspected probes on the exterior. Probes to the interior of the husk were smaller in circumference and easily separated and counted. In maturity class 4 nuts, probes were difficult to discern on the interior husk because the red dye did not stand out against the deep chocolate brown coloring characteristic of the interior husk at this stage. Therefore, the interior husk is ideal for counting probes to class 1, 2, and 3 nuts but in class 4 nuts, probes are more easily identified on the exterior husk. A significant positive relationship was found between the number of interior husk probes and the number of kernel pits, in class 1, 2, and 3 nuts (Fig. 4A–C). In class 4 nuts, a significant positive relationship was found between the number of exterior husk probes and the number of kernel pits (Fig. 5).

The failure rate in predicting kernel damage from stained probe marks on the husk and shell was low (Table 2). Using stained probe marks on the external husk only resulted in failure rates ranging from 0 to 6% among the four maturity classes. Failure rates could be improved slightly by examining the husk interior in

Table 2. **Failure rate when predicting kernel damage from *N. viridula* husk probes in macadamia nuts**

Maturity class	n^a	Failure rate: Overall % kernel damaged		No exterior husk probe but kernel damaged (%)		No exterior or interior husk probe but kernel damaged (%)	
		Mean	SEM	Mean	SEM	Mean	SEM
1	600	13.0ab	2.35	3.94	2.59	0	0
2	1,000	9.6b	3.80	0.51	0.51	0.51	0.51
3	800	23.7a	7.75	0	0	0	0
4	3,600	21.9a	2.76	5.98	1.79	4.30	1.16

Means within a column followed by the same letter are not significantly different by (P > 0.05) according to a Student's *t*-test.
[a] Number of nuts sampled.

Table 3. Frequency of macadamia nut husk probing by *N. viridula* without penetration to the kernel

Maturity class	n[a]	Overall undamaged kernel (%)		External husk probed but kernel undamaged (%)		External and internal husk probed but kernel undamaged (%)	
		Mean	SEM	Mean	SEM	Mean	SEM
1	600	87.0	3.23	7.20	2.93	4.73	2.48
2	1,000	91.4	3.47	3.45	1.39	2.21	1.06
3	800	76.3	7.89	7.87	1.72	6.04	2.05
4	3,600	78.1	3.22	5.88	1.05	3.43	0.78

[a] Number of nuts sampled.

addition to the husk exterior for evidence of probes. Using probe marks to the external and internal husks resulted in failure rates between 0 and 4.3% among the four maturity classes (Table 2). Failure rates were highest in mature (class 4) nuts for the reasons mentioned above (dark brown color of the interior husk, husk russeting). These results demonstrate that *N. viridula* feeding damage can be reliably detected in young, developing nuts and in mature nuts by using the dye technique. Cracking the shell and drying the kernel to examine dark pits is useful for more mature nuts but not for immature nuts because drying turns immature nuts entirely brown.

N. viridula infrequently probes the exterior and interior husk of macadamia nuts without penetrating through to the shell or the kernel (Table 3). Across all nut maturity classes, 5.9–7.9% of nuts with probes to the external husk had no damage to the kernel, and 2.2–6.0% of nuts with probes to the external and internal husk had no probes to the kernel. Multiple stink bugs probes into macadamia nuts and multiple pits to the kernel were common (Fig. 4). One heavily damaged macadamia nut had 120 stained probe marks to the exterior of the husk and 30 distinct pits on the kernel (Fig. 5).

N. viridula can begin probing and feeding on nuts in the tree canopy as soon as nuts are set, as evidenced by the probing to class 1 nuts. Other immature nut stages (classes 2 and 3) collected directly from the tree also showed probing activity and kernel damage, confirming that significant damage occurs in the canopy during nut development. Currently, almost all information on *N. viridula* and other insect damage to macadamia nuts comes from quality control analysis of harvest samples (class 4 nuts) at the processor. Our data indicate *N. viridula* may be damaging nuts as much as 6 mo before harvest (Fig. 2), and information from processor samples is usually not available until a month or more after harvesting begins. Therefore, making management decisions in a timely manner is impossible because of the lag time.

To successfully apply an integrated pest management (IPM) program, an effective method to monitor population levels is needed so that outbreaks can be anticipated and management decisions made in response. Traps and pheromones are often used to monitor pest population trends in other systems, but there are currently no commercially available traps or pheromones lures for *N. viridula.* As an alternative, *N. viridula* activity can be monitored indirectly by staining nuts for signs of probing. The ruthenium red dye technique stains *N. viridula* feeding probes in both immature and mature nuts, thus allowing evaluation of *N. viridula* feeding activity throughout the year. Surveying orchards using the dye will help track changes in feeding activity and identify the specific location of outbreaks. The data showed that staining of probes in the husk is highly correlated with kernel damage. Therefore, we have a rapid method to track *N. viridula* activity and forecast potential outbreaks. The technique is simple, can be learned rapidly, and is easily used by growers. Future studies using the dye technique will focus on off-season feeding activity of *N. viridula* on various cultivars in macadamia orchards. The dye also may be of use in evaluating the effectiveness of *N. viridula* management tactics such as weed control (mowing or herbicide), insecticide sprays, and augmentative biological control.

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